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MMP-13 predominates over MMP-8 as the functional interstitial collagenase in mouse atheromata

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METHODS

Animal Preparation

We studied the impact of MMP-13 and MMP-8 inhibition on atherosclerosis-susceptible *apoE*$^{-/-}$ mice with congenic c57bl/6 background (Jackson Laboratory, Bar Harbor, ME). MMP-13$^{-/-}$ and MMP-8$^{-/-}$ mice were generated by embryonic gene targeting and crossed with apoE$^{-/-}$ mice to generate respectively MMP-13$^{-/-}$ apoE$^{-/-}$ and MMP-8$^{-/-}$ apoE$^{-/-}$ double knockout (DKO) mice, as previously described[1, 2]. Dr. Stephen Krane (Massachusetts General Hospital, Boston, MA) generously provided the double knockout MMP-8$^{-/-}$MMP-13$^{-/-}$ mice. Crossing MMP-8$^{-/-}$ MMP-13$^{-/-}$ mice with apoE$^{-/-}$ animals allowed us to generate triple heterozygous, then MMP-13$^{-/-}$ MMP-8$^{-/-}$ apoE$^{-/-}$ triple knockout (TKO) mice, once bred together. 8-to-10-week-old male mice consumed an atherogenic diet (semi-purified chow containing 1.25% cholesterol and 0% cholate, Research Diets, New Brunswick, NJ) for either 10 weeks or 24 weeks. All mice were maintained in animal facilities at Harvard Medical School. Animal care and procedures were reviewed and approved by the Institutional Animal Care and Use Committees and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Lipid and weight measurements

Total body weight was measured weekly after the introduction of high-fat diet. We collected blood samples by cardiac puncture and isolated plasma after centrifugation. We measured triglycerides and cholesterol levels using kits (Thermo Scientific, Rockford, IL), according to the manufacturer's specifications.

Characterization of Aortic Lesions

Mouse aortae from the aortic arch to the iliac bifurcation were dissected and cleaned free of connective and adipose tissues. The isolated aortas were placed in 10% neutral buffered formalin overnight. We further assessed the extent of aortic atherosclerotic lesions by performing en face staining with oil red O.

Histological Assays

For histological evaluations of the aortic root, we used the method of Paigen et al.[3] Briefly, hearts were dissected in the region of the proximal aorta, and aortic roots were embedded in optimum cutting temperature compound (OCT, Sakura Finetek, Netherlands). We performed immunohistochemistry studies using rat anti-mouse monoclonal antibody for Mac3, a macrophage marker (BD PharMingen, San Diego, CA), and smooth-muscle cell (SMC) α-actin staining with primary antibody FITC-conjugated α-actin mouse monoclonal (Sigma-Aldrich, St. Louis, MO), followed by anti-FITC biotin–conjugated secondary antibody (Sigma-Aldrich), and counterstained with hematoxylin (Sigma-Aldrich). For quantification of histological assays, captured photomicrographs were analyzed with an image analysis system (ImagePro Plus 5.1, Media Cybernetics, Rockville, MD). Similarly, brachiocephalic arteries were embedded in OCT and 6-μm sections were stained for SMC α-actin and counterstained with hematoxylin. We investigated for the presence of buried fibrous caps, characterized by SMC-rich layers invested with elastin and usually overlying foam cells, as described by Jackson et al. [4] We evaluated the necrotic core by measuring the area of hematoxylin-negative acellular areas in the intima.[5, 6]

Collagen fiber characterization
We performed quantitative analysis of fibrillar collagen content using picrosirius red staining of sections viewed under polarized light[1, 7]. Qualitative analysis of fiber thickness was assessed using green and red optic filters (HQ535/50m, D605/55m, Chroma, Bellows Falls, VT) disposed under polarized light. Fiber color variation progresses from green to red proportionally to the increase of fiber thickness, such that red represents thicker, larger fibrils. The relative amount of each fiber color was expressed as a percentage of the total amount of collagen in the region. Images were recorded by a digital camera (DS-U2, Nikon, Tokyo, Japan) mounted on a polarizing microscope (Nikon Eclipse 80i), and analyzed using image analysis software (ImagePro Plus).

In situ zymography
To assess MMP collagenase activity *in situ*, we incubated 6-µm aortic root sections with DQ-Collagen substrate (Invitrogen, Carlsbad, CA) in agarose 1%, in the presence of the broad-spectrum MMP inhibitor Ilomastat (10 µM, EMD Millipore, Billerica, MA). Quantification of the fluorescence intensity in the intima (mean fluorescence intensity) reflected the collagenolytic activity.

Statistical Analyses
Continuous variables are summarized as mean ± SEM. Two investigators performed the analyses blindly. Data were analyzed by the Kruskal–Wallis one-way analysis of variance and the Bonferroni post test for each factor at individual times (Prism, GraphPad Software, La Jolla, CA). Differences were considered statistically significant at the $p<0.05$ level.

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